The effect of immobilization of heparin and bone morphogenic protein-2 (BMP-2) to titanium surfaces on inflammation and osteoblast function

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The aim of this study was to investigate biologic function of bone morphogenetic protein-2 (rhBMP-2) immobilized on the heparin-grafted Ti surface. Ti surfaces were first modified by 3-aminopropyltriethoxysilane (APTES), followed by grafting of heparin. BMP-2 was then immobilized on the heparin-grafted Ti surfaces. Pristine Ti and functionalized Ti surfaces were characterized by X-ray photoelectron spectroscopy (XPS), measurement of water contact angles, and protein adsorption. The biological activity of MG-63 cells on pristine and functionalized Ti surfaces was investigated by cell proliferation assays, measurement of alkaline phosphate (ALP) activity, and determination of calcium deposition. Anti-inflammatory effects were assessed by RT-PCR to measure the transcript levels of IL-6 and TNF-\textalpha. XPS revealed that heparin and BMP-2 were successfully grafted and immobilized on the Ti surfaces, respectively. In addition, Ti surfaces with BMP-2 immobilized were more hydrophilic than pristine Ti. Furthermore, BMP-2 immobilized Ti promoted significantly higher ALP activity and calcium deposition by MG-63 cells than pristine Ti. The inflammatory response was also decreased when cells were grown on heparin-grafted, BMP-2-immobilized Ti surfaces. The results of this study suggest that by grafting heparin and immobilizing BMP-2 on Ti surfaces, inflammation can be inhibited and osteoblast function promoted.

1. Introduction

Titanium (Ti) is widely used in the field of orthopedic and dental implants because of its good biocompatibility, superior mechanical properties, and excellent corrosion resistance. Because titanium prevents direct bone-to-bone contact, a variety of surface modifications have been developed to facilitate bone tissue responses toward titanium surfaces including hydroxyapatite coating, biomolecular immobilization, and control of the surface topography [1–3]. In recent years, another approach to stimulate bone—titanium interactions has been developed; this approach involves immobilizing peptides, such as Arg—Gly—Asp (RGD) and lysine—arginine—serine—arginine (KRSR), on the Ti surface [4–6]. However, the adhesion layer between the titanium and these peptides is weak and tends to crack. To solve these shortcomings, another approach involving surface modification with bone morphogenic proteins (BMPs) has been developed. BMPs play important roles in bone and cartilage regeneration. Among BMPs, BMP-2 has very strong osteoinductive activity. Previous studies have shown that BMP-2 can induce the osteogenic differentiation of mesenchymal cells and de novo orthotopic or ectopic bone formation [7–10]. Although BMP-2 has successfully been used to stimulate bone regeneration, use of BMP-2 is expensive, a high dose is required (1 mg BMP-2/mL defect), and BMP-2 has a short half-life in vivo [11]. To overcome these problems, BMP-2 delivery systems such as collagen gels, sponges, scaffolds, hyaluronic acid, and fibrin gels for prolonged, local release of BMP-2 have been studied [12–19]. However, these systems have problems such as uncontrolled release rates, release of BMP-2 for only a short period, and a high initial burst of release [20]. In this study, we used heparin to control the release of BMP-2. Heparin, a highly sulfated and linear natural polysaccharide, has been shown to have binding affinities to various growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming...
growth factor-
(TGF-β) [21]. Moreover, biomaterial systems with heparin have been shown to have advantages for the controlled release of these growth factors [22,23]. However, inflammatory responses from the patient’s own skin and/or mucosa and the implant materials sometimes occur during surgical insertion. With the popularization of dental implants, the incidence of peri-implant disease is now a growing problem causing local bone destruction resulting in failure of the implants. For the above reasons, implant surfaces should have both anti-inflammatory activity and facilitate biomolecular adhesion to enhance osteoblast function. Therefore, in this in vitro study, we modified the surface of titanium using heparin. Heparin has well characterized anti-inflammatory and anticoagulant properties [24]. According to Heana et al., low-molecular-weight heparin (enoxaparin) reduced the high glucose-induced activation of endothelial cells by inhibiting monocyte adhesion [25]. In this study, the free amino groups of 3-aminopropyltriethoxysilane (ATPES) were first anchored to the titanium surface to create regions of high positive charge, and then heparin was covalently grafted. Heparin was grafted to the titanium surface using a 1-3-ethyl-3-dimethylaminopropyl carbodiimide (EDC)-mediated coupling reaction between the primary amine groups of rhBMP-2 and the carboxyl groups of heparin.

We hypothesized that a Ti surface with grafted heparin and immobilized rhBMP-2 would inhibit inflammation and enhance osteoblast function.

2. Materials and methods

2.1. Amino-functionalized titanium

Titanium (Ti) discs were kindly supplied by Dio Implant Co., Ltd (Busan, Korea). Ti discs were washed in an ultra-sonicator containing absolute ethanol for 1 h prior to use. Amino-functionalized Ti discs were produced in anhydrous toluene containing 3-aminopropyltriethoxysilane (ATPES, Sigma–Aldrich, MO, USA). In brief, 50 Ti discs were immersed in 250 mL anhydrous toluene and 10 mL (v/v) of ATPES. The reaction was performed at 120°C under an N2 atmosphere with a reflux condenser for 24 h. Ti discs were then washed with toluene for 20 min to remove unreacted silane. This washing procedure was repeated five times. After washing, the amino-functionalized Ti discs were dried at 60°C for 24 h.

2.2. Grafting of heparin and immobilization of recombinant human bone morphogenetic protein-2 (rhBMP-2) to titanium surfaces

Heparin was grafted to the surfaces of the amine-treated Ti discs by the 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC)-mediated reaction between the primary amine groups of the Ti surface and the carboxyl groups of heparin. Briefly, 1 mg/mL heparin was dissolved in 0.29 mg EDC/0.18 mg NHS in 0.1 M MES buffer (pH 5.6). The Ti discs were then immersed in the aforementioned solution for 24 h at room temperature. After the reaction, the Ti discs were thoroughly washed with 0.1 M Na2HPO4 (1 h), 4 M NaCl (three times in 24 h), and distilled water (three time in 24 h), respectively. After washing, the Ti discs were frozen at −80°C for 24 h and lyophilized for 3 days. rhBMP-2 at a concentration of 0.5 or 50 ng/mL was immobilized on the surface of the heparin-grafted Ti discs. In brief, the heparin-grafted Ti discs were immersed in 0.1 M MES buffer solution (pH 5.6). rhBMP-2 at a concentration of 10 or 50 ng/mL was then added to the 0.1 M MES buffer solution (pH 5.6) and the reaction was allowed to proceed for 24 h at room temperature.

2.3. Determination of the amount of heparin-grafted to the titanium surface

The amount of heparin-grafted on the surface of the titanium discs was determined using the toluidine blue method. Briefly, Ti discs were placed into 1 mL phosphate buffer saline (PBS, pH 7.4) solution containing 1 mL of 0.005% toluidine blue solution. After 30 min under gentle shaking, 2 mL of hexane was added. After the Ti discs were removed from solution, the absorbance of the aqueous phase was measured at 620 nm. The amount of heparin coated on the surface of titanium was calculated from a calibration curve that was constructed using various concentrations of heparin.

2.4. In vitro rhBMP-2 release study

To evaluate the release kinetics of rhBMP-2 immobilized on the surfaces of heparin-grafted Ti discs, the Ti discs were soaked in a 15 mL conical tube (Falcon, USA) containing 3 mL PBS (pH 7.4) at 100 rpm at 37°C. At predetermined time intervals of 1 h, 2 h, 8 h, and 1, 3, 5, 7, 14, 21, and 28 days, the supernatant was collected and replaced with fresh PBS solution. All samples were stored at −20°C until analysis. The absorbance of samples was determined with an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions using a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 495 nm.

2.5. Characterization of the Ti discs

To determine the surface morphologies of pristine and functionalized Ti discs (heparin-grafted Ti discs and BMP-2-immobilized Ti discs), a scanning electron microscope (SEM, S2300, Hitachi, Japan) was used. The substrates were coated with gold using a sputter-coater (Eiko IB, Japan). The SEM was operated at 15 kV. The surface composition of pristine Ti discs, heparin-grafted Ti discs, and rhBMP-2-immobilized Ti discs was analyzed by X-ray photoelectron spectroscopy (XPS) on a K-Alpha spectrometer (Thermo Electron, USA) with an Al Kα X-ray source (1486.6 eV photons). The Cs1 hydrocarbon peak at 284.8 eV was used as the reference for all binding energies. The area of each peak was normalized to the total peak area of all atomic elements to calculate surface atomic percentages. To evaluate the hydrophilic properties of pristine and functionalized titanium surfaces, contact angles were measured using the sessile drop method and a video contact angle instrument (Phoenix 150, SEO, Korea) at room temperature.

2.6. In vitro cellular responses

MG-63 cells (human osteosarcoma cell line, Korean Cell Bank Line, Seoul, Korea) were used to characterize the biocompatibility of the pristine and functionalized Ti discs (heparin-grafted Ti discs, rhBMP-2 (10 ng)-immobilized Ti discs, and rhBMP-2 (50 ng)-immobilized Ti discs) by measuring cell proliferation, alkaline phosphatase activity, and calcium deposition. Cells were cultured in α-100 culture plates at 37°C in a humidified atmosphere supplied with 5% CO2. Cells were maintained in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% FBS, 50 µg/mL ascorbic acid, 10 mM dexamethasone, and 10 mM β-glycerolphosphate in the presence of 100 mM penicillin and 100 µg/mL streptomycin. Prior to cell-seeding, specimens were sterilized with 70% EtOH for 10 min and rinsed twice with phosphate-buffered saline (PBS).

2.7. Cytotoxicity test

Cytotoxicity tests for pristine and functionalized Ti discs were carried according to the ISO/EN 10993 Part 5 guidelines. To obtain extraction media, DMEM medium was incubated with pristine, amine-treated Ti, and functionalized Ti discs, respectively, for 24 h at 37°C. NH3TT3 Fibroblasts and MG-63 cells were seeded into 96-well plates at a concentration of 5 × 103 cells/well and incubated for 24 h at 37°C with DMEM supplemented with 10% FBS, and 1% 100 µg/mL penicillin and 100 µg/mL streptomycin. After 24 h of culture, the DMEM medium was removed from the 96-well plates, the cells were washed with PBS, and extraction media was added. Cells were incubated for 24 and 48 h. At each time point, the extraction medium was aspirated and CCK-8 proliferation kit (Dojindo, Japan) reagents were added to cells. Cells were then incubated for 1 h at 37°C, and the optical density of live cells was measured using a microplate reader at a wavelength of 450 nm.

2.8. Live/dead assay

The viability of cells on the surface of the pristine and functionalized Ti discs was assessed by live/dead staining. In brief, MG-63 cells were seeded at a density of 5 × 104 cells/mL on the surface of a series of Ti discs in 48-well plates. After a 48 h incubation, cells/specimens were rinsed three times with PBS and then incubated with live/dead stain (2 µM calcein AM and 4 µM ethidium homodimer-1) for 30 min at room temperature (RT). Viable cells (green) and dead cells (red) were counted under a confocal laser scanning microscope (CLSM, E2-C1, Nikon, Japan).

2.9. Protein adsorption assay

To evaluate protein adsorption on the surfaces of pristine and functionalized Ti surfaces, fibronectin and bovine serum albumin (BSA) were used as representative proteins. Five hundred microliters of BSA (1 mg/mL BSA/PBS) or fibronectin (1 mg/mL fibronectin/PBS) were pipetted onto each surface, respectively. After 3 h, non-adherent proteins were removed and collected. Bradford solution (Bio-Rad, Hercules, CA, USA) was added to each surface for 1 h at 37°C. Protein concentrations were determined using the Bradford assay according to the manufacturer’s protocols, and absorbance was measured using a microplate reader at a wavelength of 595 nm.

2.10. Cell proliferation

MG-63 cells were seeded on pristine and functionalized Ti discs at a density of 1 × 104 cells and incubated for 7 days. At predetermined time intervals (1, 3, and 7 days), specimens were rinsed with PBS and CCK-8 proliferation kit reagents were added to the specimens. After a 1 h incubation, reagents were carefully transferred...
The optical density was measured using a microplate reader at a wavelength of 450 nm.

2.11. Alkaline phosphate (ALP) activity assay

After 7, 14 and 21 days of culture, ALP activity was measured as described previously with some modifications. In brief, cells were seeded at a density of \(1 \times 10^5 \text{cells/ml} \) on pristine and functionalized Ti discs. The cells were washed with PBS and then 1X RIPA buffer ([50 mM Tris—HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA including protease and phosphatase inhibitors (1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 \(\mu\)g/mL aprotinin, 1 \(\mu\)g/mL leupeptin, 1 \(\mu\)g/mL pepstatin)]) was added to cells. The cells in RIPA buffer were sonicated using a Vibra Cell® instrument (Sonics & Materials INC., Danbury, CT, USA) for 1 min at 110 W on ice. After sonication, the cell lysates were centrifuged at 13,500 rpm for 4 \(\text{min} \) to remove cell debris. The supernatants were incubated with p-nitrophenyl phosphate solution for 30 \(\text{min} \) at 37 °C. The reaction was stopped by adding 500 \(\mu\)l of 1 N NaOH. ALP activity was determined by measuring the conversion of p-nitrophenyl phosphate to p-nitrophenol. Optical density was determined by using a microplate reader at a wavelength of 405 nm.

2.12. Calcium deposition

To quantify calcium deposition, cells were seeded at a density of \(1 \times 10^5 \text{cells/ml} \) on pristine and functionalized Ti discs. At predetermined culture time intervals (7, 14 and 21 days), cells were washed with PBS and gently scraped off from the surface of the substrates. Cells were harvested by centrifugation at 13,500 rpm for 1 \(\text{min} \), and lysis buffer (0.1% Triton X-100) was then added to the cells. The cells in RIPA buffer were sonicated using a Vibra Cell® instrument (Sonics & Materials INC., Danbury, CT, USA) for 1 min at 110 W on ice. After sonication, the cell lysates were centrifuged at 13,500 rpm for 4 \(\text{min} \) to remove cell debris. The supernatants were incubated with p-nitrophenyl phosphate solution for 30 \(\text{min} \) at 37 °C. The reaction was stopped by adding 500 \(\mu\)l of 1 N NaOH. ALP activity was determined by measuring the conversion of p-nitrophenyl phosphate to p-nitrophenol. Optical density was determined by using a microplate reader at a wavelength of 405 nm.

2.13. Anti-inflammatory effects

Murine macrophage cells from the cell line RAW 264.7 (Korean Cell Lines Bank, Seoul, Korea) were seeded on the surface of pristine Ti discs (negative control), heparin-grafted Ti discs, BMP-2 (10 ng/mL)-immobilized Ti discs, BMP-2 (50 ng/mL)-immobilized Ti discs, and LPS (100 ng/mL)-induced Ti discs (positive control) at a density of \(5 \times 10^4 \text{cells} \). After a 24 h incubation, cells were harvested for total RNA isolation. Isolated total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, CA, USA) and 1 \(\mu\)g of total RNA was reverse transcribed into cDNA using AccuPower RT PreMix (Bioneer, Daejeon, Korea) according to the manufacturer’s protocols. All PCR amplifications were performed using AccuPower PCR PreMix (Bioneer, Daejeon, Korea). The primers for TNF-α were 5'- GCC AGG TCT ACT TTG GAG TCA TTG C - 3' (sense) and 5'- AAT TTG GCT CCA GAT CTT GTC A - 3' (antisense), the primers for IL-1β were 5'- GTG TGG TGG TGA AAT CCA CTA G - 3' (sense) and 5'- AGG AGG CTT GGA CCT CTC TAT C - 3' (antisense), the primers for IL-6 were 5'- ACT GTG TCA AGG TCT TTT CCA CTG C - 3' (sense) and 5'- TCT GAA CTT CAT TGA TG T - 3' (antisense). The PCR cycling profile was as follows: 30 s at 94 °C, 1 min at 65 °C, and 1 min at 72 °C for 35 cycles after an initial denaturation step for 5 \(\text{min} \) at 94 °C. PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining.

2.14. Statistical analysis

Data are presented as means ± standard deviations. Statistical comparisons were carried out using one-way ANOVA tests. Statistically significant values are defined as *P < 0.001.

3. Results

3.1. Surface characterization

The surface morphologies of pristine and functionalized Ti discs were determined by scanning electron microscopy (Fig. 1a–d). The pristine and functionalized Ti surfaces had similar morphologies. To assess the wettability of the Ti surfaces at various stages of surface functionalization, contact angles were measured. As shown in Table 1, the mean ± standard deviation of the contact angle on pristine Ti, heparin-grafted Ti, rhBMP-2 (10 ng)-immobilized Ti, and rhBMP-2 (50 ng)-immobilized Ti was 84.18 ± 3.29, 52 ± 0.9, 48.73 ± 0.56, and 41.72 ± 0.34, respectively. To determine the chemical composition of the surfaces of pristine Ti and functionalized Ti, XPS analyses were conducted. The XPS wide-scan spectra of pristine Ti and functionalized Ti (amine-treated Ti, heparin-grafted Ti, and

Table 1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Contact angle (°)</th>
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<tbody>
<tr>
<td>Titanium (Ti)</td>
<td>84.18 ± 3.29</td>
</tr>
<tr>
<td>Heparin-Ti</td>
<td>52.22 ± 0.09</td>
</tr>
<tr>
<td>BMP-2 (10 ng)</td>
<td>48.73 ± 0.56</td>
</tr>
<tr>
<td>BMP-2 (50 ng)</td>
<td>41.72 ± 0.34</td>
</tr>
</tbody>
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rhBMP-2-immobilized Ti) and their corresponding surface elemental composition are shown in Fig. 2 and Table 2, respectively. Successful grafting of heparin to the Ti surface was indicated by an increase in the C content and a decrease in the N content compared to the amine-treated Ti surface, as shown in Table 2. Furthermore, successful immobilization of rhBMP-2 on the Ti surface was demonstrated by a decrease in the C content and an increase in the N content compared to the heparin-grafted Ti surface (Table 2). To quantify the amount of heparin-grafted to the Ti surface, toluidine blue dye was utilized. The amount of surface-exposed heparin was 0.06 μg/sample.

3.2. In vitro rhBMP-2 release study

The bioactivity of two different concentrations of rhBMP-2 immobilized on heparin-grafted Ti was analyzed by using enzyme-linked immunosorbent assays (Fig. 3). The amount of rhBMP-2 (10 ng) and rhBMP-2 (50 ng) released on the first day was approximately 69% and 26%, respectively. Over a period of 28 days, 6.9 ± 0.51 and 13.0 ± 1.38 ng of the rhBMP-2 was released from the 10 ng-rhBMP-2-immobilized titanium surfaces and 50 ng-rhBMP-2-immobilized titanium surfaces, respectively. Examination of the release curves revealed that the rhBMP-2-immobilized titanium surfaces exhibited sustained release of different concentrations of rhBMP-2 according to the starting concentration of rhBMP-2.

3.3. Protein adsorption

The protein adsorption results for pristine and functionalized Ti discs are shown in Fig. 4. Significant amounts of albumin and fibronectin adsorbed to Ti surfaces with rhBMP-2 immobilized compared to pristine Ti surfaces (P < 0.001). However, the amounts of albumin and fibronectin adsorbed on the heparin-grafted Ti surfaces were not significantly different to those adsorbed to pristine Ti surfaces. Furthermore, significantly higher amounts of albumin and fibronectin were adsorbed on the rhBMP-2 (50 ng)-immobilized Ti surfaces than the rhBMP-2 (10 ng)-immobilized Ti surfaces (P < 0.001).

3.4. Cytotoxicity tests and live/dead staining

We performed cytotoxicity tests against NIH3T3 fibroblasts and MG-63 cells for all groups the pristine Ti, amine-treated Ti, heparin—g—Ti, rhBMP-2 (10 ng)—Ti, and rhBMP-2 (50 ng)—Ti discs prior to osteoblastic cell inoculation. No significant cytotoxic effects were observed for culture periods of up to 48 h (data not shown). In addition, we assessed the cytotoxicity of pristine and functionalized Ti using a fluorescence staining method (live/dead assay). Live cells and dead cells were fluorescently labeled green and red, respectively. As shown in Fig. 5, almost all cells were alive after a 48-h exposure to either pristine Ti or functionalized Ti.

3.5. Cell proliferation

The cell proliferation of MG-63 cells on pristine and functionalized Ti was investigated at days 1, 3, and 7 (Fig. 6). Proliferation of MG-63 cells on both pristine and functionalized Ti increased slightly throughout the incubation period for up to 7 days. Cell proliferation on heparin-grafted Ti and rhBMP-2-immobilized Ti surfaces was not significantly different to that on pristine Ti surfaces.

Table 2.

<table>
<thead>
<tr>
<th>substrate</th>
<th>C% ± SE</th>
<th>N% ± SE</th>
<th>Ti% ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titanium (Ti)</td>
<td>77.45 ± 0.98</td>
<td>1.68 ± 0.37</td>
<td>20.86 ± 0.61</td>
</tr>
<tr>
<td>Amin-treated Ti</td>
<td>65.22 ± 0.48</td>
<td>16.35 ± 0.41</td>
<td>18.42 ± 0.83</td>
</tr>
<tr>
<td>Heparin-grafted Ti</td>
<td>74.64 ± 0.53</td>
<td>8.26 ± 0.27</td>
<td>17.28 ± 0.52</td>
</tr>
<tr>
<td>BMP-2 immobilized Ti</td>
<td>71.51 ± 0.59</td>
<td>10.45 ± 0.32</td>
<td>18.52 ± 0.93</td>
</tr>
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</table>

Fig. 2. XPS wide-scan spectra of (a) pristine Ti, (b) amine-treated Ti, (c) heparin-grafted Ti, and (d) rhBMP-2-immobilized Ti.

Fig. 3. Release kinetics of rhBMP-2 (10 ng) and rhBMP-2 (50 ng) from heparin-grafted Ti.

Fig. 4. Protein adsorption of pristine Ti, heparin-grafted Ti, and BMP-2 immobilized Ti after 3 h treatments of model proteins (P < 0.001).

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surfaces. There were no significant differences after 7 days of culture between cells grown on functionalized Ti and those grown on pristine Ti in terms of cell proliferation.

3.6. ALP activity

ALP is widely used as a marker of early differentiation of osteoblasts; we measured the ALP levels of MG-63 cells after 7, 14 and 21 days of culture on pristine Ti and functionalized Ti discs. The results in Fig. 7 clearly show that MG-63 cells cultured on rhBMP-2-immobilized Ti discs had significantly higher ALP activity than those cultured on pristine Ti discs (*P < 0.001). MG-63 cells grown for 7 or 14 days on rhBMP-2-immobilized Ti discs had significantly higher ALP activity than those cultured on pristine Ti discs (*P < 0.001). However, by 21 days, the ALP activity of MG-63 cells cultured on all substrates was slightly higher than the ALP activity of cells grown on all substrates at 14 days. The ALP activity of MG-63 cells grown on heparin-grafted Ti was not significantly different compared to the ALP activity of MG-63 cells cultivated on pristine Ti discs for 7, 14 or 21 days.

3.7. Mineralization

Calcium mineral deposition is a marker of the late differentiation of osteoblasts. The amount of calcium deposition that occurred after cultivating MG-63 cells for 7, 14 and 21 days on pristine and functionalized Ti discs is shown in Fig. 8. The amount of calcium deposition on all substrates increased through culture time. There were no significant differences in calcium deposition between cells grown on pristine Ti discs and those grown on heparin-grafted Ti discs. In contrast, calcium deposition on the rhBMP-2-immobilized Ti surfaces was significantly greater than that on pristine Ti surfaces (*P < 0.001). Furthermore, there were significant differences in calcium deposition between cells grown on rhBMP-2 (10 ng)-immobilized Ti surfaces and those grown on rhBMP-2 (50 ng)-immobilized Ti surfaces (*P < 0.001).
3.8. Anti-inflammatory tests

To determine whether functionalized Ti surfaces had an anti-inflammatory effect compared to pristine Ti surfaces, we measured the transcript levels of TNF-α and IL-6, which are genetic markers of inflammation. As shown in Fig. 9, mRNA levels of TNF-α and IL-6 were slightly up-regulated in cells grown on pristine Ti surfaces and strongly over-expressed in cells grown on LPS-induced Ti surfaces (positive control). In contrast, transcripts for TNF-α and IL-6 were not expressed in cells grown on heparin-grafted Ti, nor were transcripts of these cytokines detectable in cells grown on rhBMP-2-immobilized Ti surfaces (Fig. 9). These data indicate that heparin-grafted and rhBMP-2-immobilized Ti surfaces have anti-inflammatory effects compared to pristine Ti surfaces.

4. Discussion

The clinical success of dental implants is highly dependent on the cell adhesion, matrix production, anti-inflammatory, and mineralization properties of the implant materials. In the present study, we demonstrated that modification of Ti surfaces with heparin and rhBMP-2 had an anti-inflammatory effect on MG-63 cells and resulted in an increase in osteogenic activity. The rhBMP-2 (50 ng)-immobilized Ti discs showed a significantly smaller water contact angle than pristine Ti discs, indicating that the immobilized rhBMP-2 disc surfaces were more hydrophilic than those of pristine Ti. This is consistent with a study by Zhiong and co-workers [2], who reported that rhBMP-2 immobilized carboxymethyl chitosan (CMCS)-grafted Ti surfaces were more hydrophilic than pristine Ti. The chemical compositions of the surfaces after modification with heparin and rhBMP-2 were determined by XPS. Successful grafting of heparin to the Ti surface was indicated by both an increase in C and N content and a decrease in Ti content compared to Ti surfaces after ATPES processing. Moreover, successful anchoring of rhBMP-2 to the heparin-modified Ti surfaces was demonstrated by the increase in N content due to the N-containing groups in rhBMP-2 compared to the heparin-grafted Ti surfaces. A previous study reported successful immobilization of rhBMP-2 to CMCS-grafted Ti surfaces because of the formation of covalent bonds between the carboxyl groups of CMCS and the amine groups of rhBMP-2 [2]. Similarly, in our study, the carboxyl groups of heparin formed covalent bonds with the amine groups of rhBMP-2. As mentioned in the introduction, rhBMP-2 is one of the most osteoinductive growth factors, but its clinical application has been limited by its short half-life, rapid diffusion by body fluids, and the large doses required. Large doses of rhBMP-2 in particular are a problem because they can result in side-effects such as immune response and bone overgrowth from the defect site [26]. To create an effective delivery system for rhBMP-2 and ensure its release for a sufficient time period and in appropriate amounts, we modified the surfaces of Ti discs with heparin to which we immobilized rhBMP-2. We demonstrated that the rhBMP-2-immobilized heparin-grafted Ti surface was successfully released over an extended period of time. The sustained release of growth factors, including various types of matrices, from heparin has been well documented [23,27,28]. Lin et al. [28] demonstrated that heparin-crosslinked demineralized bone matrix (DBM) to which BMP-2 was bound increased the ALP activity of attached cells and the percentage of calcified tissue in vivo. Moreover, a previous study demonstrated that heparin-immobilized PLGA porous microspheres loaded with bFGF released bFGF in a more sustained manner in vitro and stimulated angiogenesis in vivo when compared to the same amount of bFGF loaded into PLGA microspheres [29]. These results demonstrate that heparin is a suitable material for the sustained release of growth factors. We observed an increase in osteoblast proliferation when osteoblasts were cultured on all substrates. However, cell proliferation on rhBMP-2-immobilized Ti discs did not lead to cell proliferation when osteoblasts were cultured on all substrates.
not show a significant increase compared to that on pristine Ti, consistent with the reports of Zhilong et al. [2,30] In contrast, Park et al. reported an increase in the proliferation of osteoblasts grown on nanofibrous chitosan membranes with immobilized BMP-2 compared to their proliferation on nanofibrous chitosan membranes without immobilized BMP-2 [31]. Thus, the effects of rhBMP-2 on osteoblast proliferation are still unclear.

ALP activity and calcium deposition are widely used as markers for early and late differentiation of osteoblast cells, respectively [32,33]. ALP activity was measured after a culture period of 7, 14 or 21 days. There was no significant difference in osteoblast proliferation between cells grown on heparin-grafted Ti versus pristine Ti discs throughout the 21 days. In contrast, the ALP activity of osteoblasts cultured on rhBMP-2-immobilized Ti surfaces was significantly higher than that of osteoblasts cultured on pristine Ti surfaces for the different culture periods. Thus, rhBMP-2 stimulates osteoblast differentiation. However, the ALP activity of osteoblasts on all substrates at 21 days was slightly lower than that on all substrates at 14 days. This means that ALP activity reached a maximum before mineralization actually began [34]. Furthermore, these results indicate that ALP activity may diminish slightly and calcium deposition may increase slowly after a culture period of 21 days. In Materials like DBM, titanium, and chitosan-immobilized BMP-2 have been shown to increase the ALP activity of osteoblast-like cells [2,28,31]. Significantly more calcium was deposited on the rhBMP-2-immobilized Ti surfaces than on pristine Ti surfaces over a culture period of 21 days. As was expected from the ALP activity profile, the amount of calcium deposited on heparin-grafted Ti surfaces was similar to that deposited on pristine Ti surfaces throughout the 21 day culture period. In contrast, there was a significant difference in calcium deposition between cells growing on rhBMP-2 (10 ng)-immobilized Ti surfaces and those growing on rhBMP-2 (50 ng)-immobilized Ti surfaces at 7, 14 and 21 days. Together, these results indicate that rhBMP-2-immobilized Ti substrates can stimulate matrix formation and enhance osteoblast cell function.

We examined the anti-inflammatory effects of pristine Ti and functionalized Ti by measuring transcript levels of the pro-inflammatory cytokine interleukin 6 (IL-6) and tumor necrosis factor (TNF-α). Expression of these cytokines was not detected in osteoblasts cultured on heparin-grafted Ti surfaces. In contrast, slight up-regulation of pro-inflammatory cytokines was detected in cells grown on pristine Ti surfaces. In an earlier paper, Thurani et al. demonstrated that heparin and O-desulfated heparin inhibited NF-κB activation in a TNF-α-stimulated human endothelial cell line and in ischemic-reperfused rat myocardium [35]. A further study showed that unfractionated heparin and low-molecular weight heparin downregulated pro-inflammatory cytokines and NF-κB in LPS-stimulated human monocytes [36]. These results suggest that heparin has anti-inflammatory activity, consistent with the results of our study.

Our present study has the following clinical implications: (i) sustained release of rhBMP-2 from rhBMP-2 immobilized, heparin-grafted Ti surfaces can be achieved over long periods of time; (ii) suitable doses of rhBMP-2 can enhance osteoblast function; and (iii) heparin-grafted Ti can decrease the inflammatory response. Regarding the current concerns on peri-implant diseases [37,38], the anti-inflammatory action of this functionalized Ti can give us possibility to reduce the incidence of peri-implant disease. Considering the prevention of peri-implantitis is one of major concerns in implant dentistry, the possible anti-inflammatory effect by this functionalization process may be promising to reduce the tissue destruction by peri-implantitis. Further studies are required to determine the appropriate doses of rhBMP-2 for in vivo application of this delivery system and validate our results.

5. Conclusions

Ti substrates were successfully functionalized via a chemical method involving grafting of heparin and subsequent immobilization of rhBMP-2. Heparin reduced inflammation effectively and sustained release of rhBMP-2 was achieved. The rhBMP-2-immobilized Ti substrates stimulated osteoblast functions successfully. Furthermore, cells grown on rhBMP-2 (50 ng)-immobilized Ti surfaces had significantly greater ALP activity and calcium contents than cells grown on pristine Ti surfaces. Thus, rhBMP-2-immobilized, heparin-grafted Ti discs are a suitable delivery system to enhance osteoblast function and decrease inflammatory reaction and may therefore be suitable for incorporation in implant materials for orthopedic and dental applications.

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Appendix

Figures with essential color discrimination. Fig. 5 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at 10.1016/j.biomaterials.2010.09.008

References


Kim SE, et al., The effect of immobilization of heparin and bone morphogenic protein-2 (BMP-2) to...